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Determination of Glutathione Peroxidase Activity and its Contribution to Hydrogen Peroxide Removal in Erythrocytes

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A new method for the determination of glutathione peroxidase activity in erythrocytes was developed. The present method was applied to the measurement of hydrogen peroxide removal rates by glutathione peroxidase in erythrocytes at 70 μM hydrogen peroxide under simulated *in vivo* conditions. The removal rates by glutathione peroxidase in mouse erythrocytes were twenty-times faster than those in human ones and were 5.2 μmol/sec/g of Hb. The removal rates in acatalasemic mouse erythrocytes indicate that glutathione peroxidase is the main means of hydrogen peroxide removal in acatalasemic mouse erythrocytes. Based on these results, we concluded that glutathione peroxidase in mouse erythrocytes had sufficient ability to remove hydrogen peroxide at even relatively high concentrations. This may be one of the reasons why acatalasemic mice suffer no health problems while Japanese acatalasemic patients suffer from Takahara disease when infected with hydrogen peroxide-generating bacteria.

Key words: glutathione peroxidase, erythrocyte, hydrogen peroxide, acatalasemic mouse, Takahara disease

To study detoxification of hydrogen peroxide by erythrocytes, we examined hydrogen peroxide removal rates in mouse hemolysates and characterized the removal activities by catalase (EC 1. 11. 1. 6) and hemoglobin in erythrocytes (1, 2). Regarding glutathione peroxidase activity (EC 1. 11. 1. 9) in erythrocytes, Maral *et al.* (3) reported that the activity in mouse erythrocytes was higher than that in human ones, but another group (4) reported that the activity was the same as that in human ones. Therefore, we chose to examine hydrogen peroxide removal rates by glutathione peroxidase in erythrocytes.

For the measurement of glutathione peroxidase activity, the standard method consists of treatment of hemolysates with Drabkin's reagent to minimize the removal activity by hemoglobin and a glutathione peroxidase assay coupled with glutathione reductase (3-6). However, the treatment with Drabkin's reagent contains tedious steps, and a few of the reagents used in the method are relatively unstable and expensive.

In this study, we developed an easily accessible method for the determination of glutathione peroxidase activity in erythrocytes using hydrogen peroxide as a substrate. We applied it to the measurement of hydrogen peroxide removal rates by glutathione peroxidase in erythrocytes under simulated *in vivo* conditions in order to estimate the actual removal ability of erythrocytes.

Materials and Methods

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Materials. *meso*-Tetrakis(1-methyl-4-pyridyl)-porphyrin was purchased from Dojindo Labs (Kumamoto, Japan), and *meso*-tetrakis(1-methyl-4-pyridyl)porphinato-iron (III) pentachloride ($FeTMPyPCl_5$) was prepared by inserting iron into *meso*-tetrakis(1-methyl-4-pyridyl)porphyrin (7). Carboxymethyl-cellulose (CM52) was purchased from Whatman (Maidstone, UK). Hydrogen peroxide and other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan). Concentration of hydrogen peroxide was determined according to the method of Nakano and Takahashi (8).

Human blood samples were obtained from healthy adult male volunteers (31-49 years old). Male mice (12-16 weeks old) of C3H/AnLCs^{n}Cs^{n} (normal) and C3H/

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AnLCs^{b}Cs^b (acatalasemia) strains were used for experiments. Mice were fed on a laboratory diet (MF) or selenium-free diet (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*, and blood samples were collected.

General methods. Washed erythrocytes from human and mouse blood were prepared as described previously (1), and hemolysate was prepared by the addition of 9 volumes of water. Hemoglobin contents were measured by the method of Drabkin and Austin (9). Hydrogen peroxide removal rates by hemoglobin and catalase were measured by the previously reported method (2).

Determination of glutathione peroxidase activity by the standard method. Hydrogen peroxide removal rate by hemolysate was measured at 1.2 mM hydrogen peroxide in the presence of 1.0 mM glutathione according to the method of Wendel (5). The rate of hydrogen peroxide removal by glutathione peroxidase-free hemoglobin, which was prepared by the previously reported method (2), was measured by replacing hemolysates with glutathione peroxidase-free hemoglobin. Glutathione peroxidase activity by the standard method was calculated as the difference between the two hydrogen peroxide removal rates.

Determination of glutathione peroxidase activity by the present method. One milliliter of freshly prepared hemolysates containing less than 6 mg of hemoglobin was applied to a carboxymethyl-cellulose column (0.7 × 6 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8). It was then eluted with the same buffer. Eluate between 1.0 and 3.0 ml was collected. The mixture (4.8 ml) consisting of 0.1 1.0 ml of the eluate, 0.1 ml of 50 mM glutathione and phosphate buffered saline (PBS: 140 mM sodium chloride in 10 mM potassium phosphate buffer at pH 7.2) containing 1 mM sodium azide (a catalase inhibitor) (5) was incubated at 37°C. To the mixture, 0.2 ml of 3.5 mM hydrogen peroxide was added to start the enzymatic reaction. At 0, 2 and 4 min, 1.0 ml of the reaction mixture was immediately put into a test tube containing 0.2 ml of 100 mM *N*-ethylmaleimide (NEM) and was mixed well to stop the enzymatic reaction. The mixture was diluted to 2.0 ml with PBS to adjust the hydrogen peroxide content to less than 70 μM, and 2.0 ml of the reagent solution [consisting of 10 volumes of 0.2 mM FeTMPyPCl$_5$ (λ max 402 nm, ε = 7.6 × 10^4, in 1 M HCl), 10 volumes of 41.2 mM *N*, *N*-dimethylaniline in 0.2 M HCl, 10 volumes of 8.56 mM 3-methyl-2-benzothiazolinone hydrogen chloride in 0.2 M HCl and 1 volume of 20 mM disodium EDTA] was added to determine the amount of hydrogen peroxide using formation of indamine dye as an indicator (λ max 590 nm, ε = 5.52 × 10^4) (1). The mixture was reacted at 25°C for 1 h, and the absorbance at 590 nm was recorded. The rate of spontaneous reaction of hydrogen peroxide with glutathione was measured by replacing the eluate with an equal volume of water. Glutathione peroxidase activity was calculated as the difference between the two hydrogen peroxide removal rates.

Measurement of hydrogen peroxide removal rates by glutathione peroxidase in erythrocytes under simulated in vivo conditions by the present method. Glutathione contents in packed erythrocytes were measured according to the method of Beutler *et al.* (10). Then, one milliliter of hemolysate containing less than 6 mg of hemoglobin was prepared to measure hydrogen peroxide removal rates by glutathione peroxidase under simulated *in vivo* conditions. Following the present method, hemolysate was applied to a carboxymethyl-cellulose column, and the eluate was collected. Hydrogen peroxide removal rates by glutathione peroxidase in the eluate at 70 μM hydrogen peroxide were measured at the same concentration of glutathione in packed erythrocytes. The spontaneous reaction rates of hydrogen peroxide with glutathione were measured by replacing the eluate with an equal volume of water. Hydrogen peroxide removal rate by glutathione peroxidase under simulated *in vivo* conditions was calculated as the difference between the two removal rates.

Results and Discussion

During the measurement of glutathione peroxidase activity in hemolysates by the standard method (5), we noticed that hydrogen peroxide removal rates by mouse hemoglobin were not inhibited by treatment with Drabkin's reagent (0.27 ± 0.03 μmol/sec/g Hb) while the removal in human hemoglobin were halted completely by this treatment (6). We improved the standard method by preparing glutathione peroxidase-free hemoglobin.

To eliminate the difficulty caused by hemoglobin and the tedious steps involved in the treatment with Drabkin's reagent, we decided to remove hemoglobin in hemolysates and then determine glutathione peroxidase activity by the hydrogen peroxide determination method using the formation of indamine dye, since the procedure is simple and the reagents used are quite stable (1).

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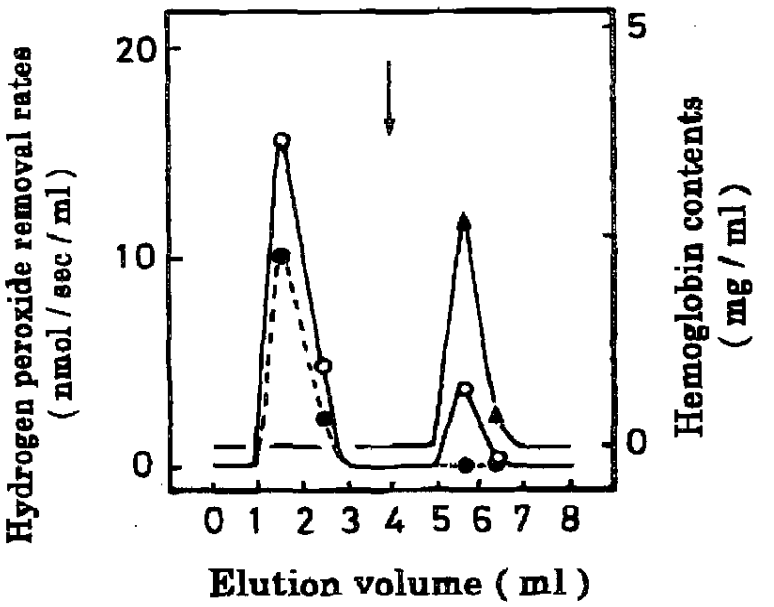

Fig. 1 Elution profile of mouse hemolysate on a carboxymethyl cellulose column. One milliliter of hemolysate prepared from normal mouse erythrocytes was applied to a carboxymethyl-cellulose column (0.7 × 6cm) equilibrated with 5mM potassium phosphate buffer (pH 6.8). The column was eluted with 3ml of 5mM potassium phosphate buffer and then 4ml of 1 M potassium phosphate buffer. Each 1.0ml of the eluate was collected. An arrow indicates the start of elution with 1 M potassium phosphate buffer. ○——○: Hydrogen peroxide removal activity by catalase and hemoglobin; ●- - ●: The activity by glutathione peroxidase; ▲——▲: Hemoglobin contents.

Passing through the small carboxymethyl-cellulose column (Fig. 1), glutathione peroxidase and catalase activities in hemolysates were eluted between 1.0 and 3.0 ml, and hemoglobin was eluted between 5.0 and 7.0 ml. From glutathione peroxidase activity in the eluate, the recovery of glutathione peroxidase from carboxymethyl-cellulose column was calculated to be 99.0 ± 2.2 % (n = 5). To detect only the hydrogen peroxide removal rates by glutathione peroxidase in the eluate, sodium azide (a catalase inhibitor) was added to the assay mixture. Reaction by glutathione peroxidase was started by adding hydrogen peroxide and was stopped by adding NEM, which removed the glutathione. Due to the presence of 1 mM sodium azide, the formation of indamine dye from hydrogen peroxide was slightly inhibited (92.1 + 0.4 % of the theoretical amount, n = 5), but the determination of hydrogen peroxide was not affected (data not shown).

To determine the hydrogen peroxide concentration for the assay of gluthathione peroxidase activity, the removal rates by mouse glutathione peroxidase in the presence of 1 mM glutathione were analyzed by a Lineweaver-Burk plot. The Michaelis constant for hydrogen peroxide was 2.90 ± 0.48 μM (n = 3), which was consistent with that reported by Flohé *et al.* (11), and the maximum velocity was 3.34 + 0.33 μmol/sec/g of Hb. Therefore, 140 μM hydrogen peroxide was chosen for the assay of glutathione peroxidase activity.

To check the accuracy of this method, we measured glutathione peroxidase activities in mouse hemolysates by the standard method and the present method (Table 1). When values (x) measured by the present method were compared with those (y) of the standard method, a regression equation of $y = 0.95x - 0.02$ with a correlation coefficient of 0.996 ($n = 8$, $P < 0.001$) was produced, indicating that the values obtained by the present method were in good agreement with those of the standard method.

Average values of glutathione peroxidase activity in human hemolysates calculated by the present method were 0.16 ± 0.07 μmol/sec/g of Hb (n = 4). The average

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Table 1 Comparison of glutathione peroxidase activity in mouse hemolysates determined by the standard method and the present method

Sample number[a]	Standard method[b] (μmol/sec/g of Hb)	Present method[c] (μmol/sec/g of Hb)
1	4.35, 4.15, 4.07	4.70, 4.58, 4.42
2	2.99, 3.04, 3.09	2.96, 3.15, 3.44
3	3.84, 3.67, 3.53	3.72, 3.79, 3.65
4	2.80, 2.82, 2.87	2.87, 3.07, 3.25
5	2.72, 2.72, 2.53	2.53, 2.64, 2.72
6	1.81, 1.78, 1.87	1.96, 2.15, 2.24
7	0.16, 0.17, 0.12	0.15, 0.18, 0.11
8	0.12, 0.09, 0.18	0.18, 0.24, 0.12

a : Samples number 1, 2, 3 and 4 were obtained from mice fed a MF diet; 5 and 6 from mice fed a selenium-free diet for 1 month; and 7 and 8 from mice fed a selenium-free diet for 12 months. Each sample was measured separately three times, and all three values were listed.

b : Activity was determined using a modification of the standard method (5). The method consisted of treatment of hemolysates with Drabkin's reagent and detection of NADPH decrease. Concentrations of hydrogen peroxide and glutathione in the assay mixture were 1.2 and 1.0 mM, respectively. Details are described in the Materials and Methods.

c : The present method consists of separation by a carboxymethyl-cellulose column and colorimetric detection of hydrogen peroxide decrease. Concentrations of hydrogen peroxide and glutathione in the assay mixture were 0.14 and 1.0mM, respectively.

values in normal and acatalasemic mouse hemolysates were 3.41 ± 0.36 ($n = 5$) and 3.45 ± 0.54 μmol/sec/g of Hb ($n = 6$), respectively. These values in human and mouse hemolysates agreed well with those reported by Maral *et al.* (3), even though they used *tert*-butyl hydroperoxide as a substrate, indicating that there was a big difference in glutathione peroxidase activity between human and mouse erythrocytes. The values in normal and acatalasemic mouse hemolysates indicate that the activities in both hemolysates were of a similar level but were different from those reported by Ogata *et al.* (4).

To examine hydrogen peroxide removal ability by glutathione peroxidase in erythrocytes, the present method was applied to measurement of the removal rates by glutathione peroxidase in erythrocytes at 70 μM hydrogen peroxide under simulated *in vivo* conditions (Table 2). The removal rates by catalase and hemoglobin at 70 μM hydrogen peroxide are also shown for comparison with the removal rates by glutathione peroxidase in erythrocytes.

The removal rates in human erythrocytes indicate that the order of contribution to detoxification of hydrogen peroxide is as follows: catalase > hemoglobin > glutathione peroxidase. This suggests that catalase activity is the main means of hydrogen peroxide removal in human erythrocytes. On the other hand, the removal rates by catalase in normal mouse erythrocytes were one quarter of those in human ones, and the removal rates by glutathione peroxidase in mouse erythrocytes were twenty-times faster than those in human ones, indicating that the removal ability by glutathione peroxidase in mouse erythrocytes was substantial. The removal rates in acatala-

Table 2 Rates of hydrogen peroxide removal by catalase, hemoglobin and glutathione peroxidase in erythrocytes at 70 μM hydrogen peroxide under simulated *in vivo* conditions

Erythrocytes (n)[a]	Removal rates of H_2O_2[b] (μmol/sec/g of Hb)
Human (4)	
Catalase	30.0 ± 4.7
Hemoglobin	1.11 ± 0.13
Glutathione peroxidase	0.24 ± 0.13
Normal mouse (6)	
Catalase	7.61 ± 0.63
Hemoglobin	1.50 ± 0.06
Glutathione peroxidase	5.19 ± 0.61
Acatalasemic mouse (6)	
Catalase	0.77 ± 0.10[c]
Hemoglobin	1.50 ± 0.15
Glutathione peroxidase	5.17 ± 0.13

a : Numbers in parentheses indicate the number of subjects.

b : Rates of hydrogen peroxide removal by catalase and hemoglobin were measured by the previously reported method (2). The rates by glutathione peroxidase at the same concentration of glutathione in erythrocytes were measured by the present method. Details are described in the Materials and Methods. Values indicated are mean ± S.D.

c : The rates were measured at 25°C because of the thermal instability of residual catalase (2).

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semic mouse erythrocytes (Table 2) indicate that the order of contribution to detoxification is as follows: glutathione peroxidase > hemoglobin > residual catalase. This suggests that glutathione peroxidase activity is the main activity of hydrogen peroxide removal in acatalasemic mouse erythrocytes. As the removal rates by acatalasemic erythrocytes at 70 μM hydrogen peroxide were substantial (1), we deduced that glutathione peroxidase in mouse erythrocytes had sufficient ability to remove hydrogen peroxide at even relatively high concentrations. This might be one of the reasons why acatalasemic mice had no associated health problems while Japanese patients with acatalasemia would suffer from Takahara disease when infected by hydrogen peroxide-generating bacteria (12).

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